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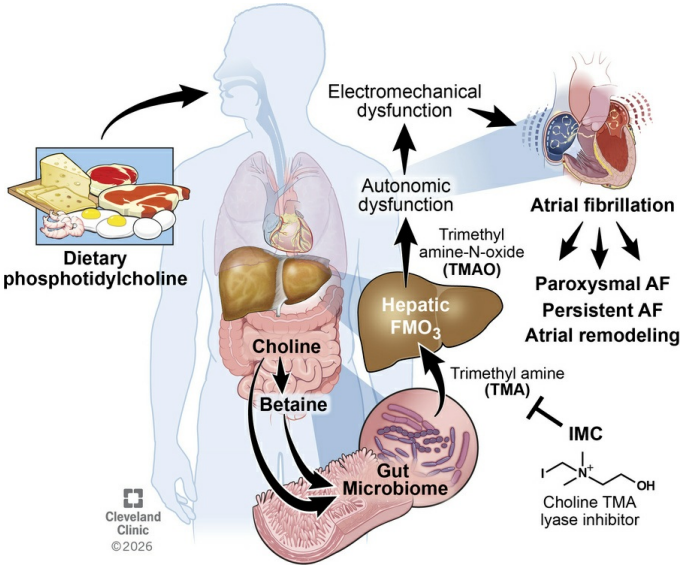
Graphical abstract



Gut microbial trimethylamine N-oxide generation promotes risk of atrial fibrillation via muscarinic receptor-mediated autonomic dysfunction

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Abstract

Gut microbiota-derived trimethylamine *N*-oxide (TMAO) plays a role in the pathogenesis of cardiovascular disease. The role of TMAO in the pathogenesis of atrial fibrillation (AF) remains uncertain. TMAO levels were quantified in plasma from serial subjects undergoing elective cardiac catheterizations (N=5090) and shown to independently associate with prevalent AF following adjustment for risk factors (TMAO adjusted odds ratio 1.7 [95% confidence interval 1.3-2.1]; $P<0.01$). Human cAMP response element modulator isoform IbΔC-X transgenic mice (CREM-IbΔC-X), a spontaneous mouse model of AF, supplemented with a TMAO diet developed AF sooner. C57BL/6J mice on and off a TMAO had more inducible AF via a transesophageal pacing study compared to chow controls. Dietary choline supplementation increased circulating TMAO levels and significantly accelerated AF onset in CREM-IbΔC-X mice ($P<0.01$). Iodomethylcholine (IMC), the gut microbial *CutC/D* inhibitor that suppresses choline→TMA(O) metabolic transformation, reduced circulating TMAO levels ($P<0.0001$) and choline induced AF onset ($P<0.01$). Cecal metagenomic analyses showed that choline supplementation induced changes in microbial communities associated with AF, while many of these changes were attenuated by IMC. Choline supplementation promoted overall adverse atrial remodeling with left atrial dilation. Optical mapping studies showed that mice supplemented with choline exhibited reduced conduction velocity, shortened action potential duration at 80% repolarization, and decreased wavelength. TMAO inhibits muscarinic receptor 2 resulting in autonomic dysfunction that promotes AF. In summary, the gut microbial metabolite TMAO, independently associated with AF risk in subjects, enhances AF in multiple AF mouse models via autonomic dysfunction, and is a therapeutic target for prevention of AF.

INTRODUCTION

The trimethylamine *N*-oxide (TMAO) pathway is mechanistically linked to the pathogenesis of numerous cardiovascular diseases (CVD)(1-3). Direct administration of TMAO, inhibition of the TMAO pathway using small molecule inhibitors(4-8) and gut microbial transplant studies(6, 9, 10) have further supported a mechanistic link between TMAO and the gut microbiome with metabolic diseases (1, 9-11). Choline is both an abundant nutrient in western diets and an endogenous component of bile. It is metabolized by the gut microbiota to yield trimethylamine (TMA), which is subsequently oxidized by host hepatic flavin monooxygenases (FMOs) to TMAO (12). TMAO has been linked to the initiation, progression, and sequelae of CVD in multiple animal and *in vitro* studies (2, 12-16). In large clinical cohort studies TMAO is independently associated with major adverse cardiovascular events and adverse heart failure outcomes (2, 17-23). Mechanistically, TMAO has been implicated in the pathogenesis of CVD by increasing foam cell formation (1), disrupting reverse cholesterol transport (24), increasing platelet hyperreactivity (25), and upregulating the endoplasmic reticulum stress response by activating the protein kinase R-like endoplasmic reticulum kinase (15). TMAO has also been implicated in the pathogenesis of heart failure and kidney disease by promoting fibrosis and inflammation, pathways also important in the etiology of atrial fibrillation (AF) (8, 26-28).

Despite advances such as advent of catheter ablation, antiarrhythmic medications, and novel oral anticoagulants, AF remains a significant cause of morbidity and mortality worldwide, and its underlying pathogenic mechanisms are not fully understood (29, 30). TMAO is involved in the pathogenesis of kidney disease and heart failure by promoting renal and cardiac fibrosis and inflammation pathways associated with an increased risk of AF (8, 16, 31). The many shared risk factors between CVD and AF suggest that there may also be a link between TMAO and AF (32). Here, we explore the relationship between TMAO and AF risk, including clinical associations and both direct testing of the effect of provision of TMAO, and examination of the impact of pharmacological targeting of the gut microbial TMAO pathway in a mouse model of AF disease.

RESULTS

Plasma TMAO concentrations independently associate with prevalent AF.

Subjects were stratified into tertiles based on increasing plasma levels of TMAO, choline, and betaine. A significant association was observed between tertiles and history of AF for all three metabolites (**Figure 1A**). Tertiles of TMAO, choline, and betaine were also significantly associated with AF risk in an unadjusted model (**Figure 1B, open circles**), after adjustment for subject characteristics, comorbidities, and laboratory values (**Figure 1B, gray circles, Model 1**), or after adjustment for subject characteristics, comorbidities, laboratory values, and kidney function (**Figure 1B, black circles, Model1 + eGFR**). Cubic spline plots of the unadjusted odds ratios demonstrated that low levels of plasma trimethylamines (TMAs - TMAO, choline and betaine) are associated with a lower likelihood of having AF and TMAs at higher plasma concentrations are associated with a higher likelihood of having AF (**Figure 1C**). Forest plots of the odds ratios (ORs) of TMAO tertiles and prevalent AF are shown for individual patient characteristics and laboratory values and similar trends were observed across all key subgroups, with no statistically significant differences were noted (**Figure 2**).

Dietary TMAO supplementation accelerates AF onset and persistence in CREM-IbΔC-X transgenic mice.

The finding that TMAO is independently associated with AF in humans suggested that it may contribute to AF pathogenesis and that inhibition of the gut microbial TMAO pathway could represent a potential preventive therapeutic approach. The CREM-IbΔC-X mouse is a model that develops spontaneous AF; it recapitulates human AF by first developing paroxysmal AF and then ultimately developing persistent AF (33, 34). Male and female CREM-IbΔC-X mice supplemented with dietary TMAO (0.3%) developed paroxysmal AF (first time onset of AF) and persistent AF (10 serial ECGs demonstrating AF) sooner compared to the chow diet using serial needle electrode ECG measurements (**Figure 3A; Supplemental Table 1 and 2**)**.** CREM-IbΔC-X mice supplemented with dietary TMAO had significantly higher plasma levels of TMAO (**Figure 3B**). Similar trends were observed for the onset of paroxysmal and persistent AF and plasma TMAs (trimethylamine containing compounds TMAO, choline, and betaine) levels in both male and female CREM-IbΔC-X mice, but female CREM-IbΔC-X mice failed to reach statistical significance for the onset of paroxysmal AF (first time AF) (**Supplemental Figures 1 and 2; Supplemental Table 3 and 4**). Notably, chow and TMAO supplemented male and female CREM-IbΔC-X mice, did not differ significantly in body weight (**Supplemental Figure 3**). This data suggests TMAO and by extension the gut microbiome has a direct role in the pathogenesis of AF.

Dietary TMAO supplementation mediated an increase in AF susceptibility in C57BL/6J mice.

In order to demonstrate that the effects of TMAO were not strain dependent, we validated the key findings of our study using an alternative mouse model of AF. We performed a transesophageal AF induction study in male and female C57BL/6J mice on and off a TMAO diet. AF inducibility was increased in C57BL/6J mice fed a TMAO-supplemented diet compared with chow-fed controls (**Figure 3C**). Notably, TMAO supplementation resulted in an 11-fold increase in AF inducibility relative to controls (**Figure 3C**). When stratified by sex, both male and female C57BL/6J mice demonstrated similarly significant increases in AF inducibility (**Supplemental Figure 4**).

Plasma TMAO concentrations in C57BL/6J mice used in transesophageal pacing studies were also significantly increased in all C57BL/6J mice on a TMAO-supplemented diet compared to chow fed controls (**Supplemental Figure 5**). Additionally, plasma levels of TMAO were significantly correlated with AF percent induction and similar trends were observed when stratified by sex, but the correlation remained significant only in C57BL/6J male mice (**Figure 3D, Supplemental Figure 6A**). No consistent significant relationship was noted between AF percent induction and other plasma TMAs (**Supplemental Figure 6B, C**).

Inhibition of the gut microbial TMAO production with the TMA lyase inhibitor iodomethylcholine (IMC) significantly delays the onset of paroxysmal and persistent AF in CREM-IbΔC-X transgenic mice.

We next sought to determine whether targeting the gut microbial TMAO pathway could provide a potential therapeutic strategy for AF. CREM-IbΔC-X mice fed a choline-supplemented diet developed paroxysmal AF and persistent AF earlier (**Figure 4, A and B; Supplemental Table 5 and 6**) than chow-fed controls. Administration of IMC, an inhibitor of microbial choline to TMA pathway (7), in animals maintained on a high-choline diet (Choline + IMC) resulted in marked suppression of choline-diet-induced AF (**Figure 4, A and B; Supplemental Table 5 and 6**). Comparable trends in the onset of paroxysmal and persistent AF were observed in each sex, but CREM-IbΔC-X female mice on choline supplemented diet did not show a significant increase in paroxysmal AF (first time AF) compared to chow controls (**Supplemental Table 7 and 8**). Notably, in chow-fed mice (choline content 0.8-0.9%) (21), IMC supplementation significantly

delayed the development of paroxysmal AF compared with controls and showed a trend toward delayed onset of persistent AF (**Figure 4, A and B; Supplemental Table 5 and 6**).

CREM-IbΔC-X mice fed a choline-supplemented diet had significantly higher plasma concentrations of TMAO (**Figure 4C**) and TMA (**Supplemental Figure 9**) compared to the chow-fed (control) mice. Dietary supplementation of choline in combination with IMC led to marked reduction in plasma concentration TMAO and TMA (**Figure 4C**, **Supplemental Figure 9**). TMAO and TMA levels were not significantly reduced in mice fed chow (control) diet in combination with IMC (**Figure 4C, Supplemental Figure 9**).

Consistent with the well-established observation that the FMO3 (the liver enzyme responsible for the oxidation of TMA to TMAO) is expressed at higher levels in female than male mice (9), CREM-IbΔC-X female mice had higher circulation TMAO than male CREM-IbΔC-X mice (**Supplemental figure 10**). This observation was also true of CREM-IbΔC-X female and male mice on a chow diet (CREM-IbΔC-X female chow TMAO: 3.1 $\pm$ 1.5 μM, CREM-IbΔC-X male chow TMAO 0.5 $\pm$ 0.24 μM). Choline plasma concentrations were higher in choline diet compared to the chow diet in both male and female CREM-IbΔC-X mice (**Figure 4C**). Betaine plasma concentrations tended to be highest in choline + IMC supplemented mice compared to the other diets (**Figure 4C and Supplemental figure 10**).

Plasma TMAO concentrations significantly predicted the earlier onset of paroxysmal AF in all male CREM-IbΔC-X mice and approached significance in female CREM-IbΔC-X mice (**Figure 5A**). Higher plasma betaine concentrations associated with later onset of paroxysmal AF in male CREM-IbΔC-X mice whereas plasma choline levels had no association with the onset of paroxysmal AF (**Figure 5A**). Higher TMAO levels predicted the earlier onset of persistent AF in both male and female CREM-IbΔC-X mice whereas plasma betaine and choline plasma concentrations did not (**Figure 5A**).

In general, CREM-IbΔC-X female mice developed AF earlier and progressed to persistent AF more rapidly than male CREM-IbΔC-X mice (**Supplemental Figures 7 and 8**). In contrast, serial needle electrode ECGs performed in WT mice fed either chow or a choline-supplemented diet revealed no onset of AF over the study period (**Supplemental Figure 11**). Notably, CREM-IbΔC-X mice in the ECG studies showed no significant differences in body weight at the time of sacrifice, and no appreciable liver pathology was observed (**Supplemental Figures 12 and 13**). To

determine whether the earlier development of persistent AF in CREM-IbΔC-X mice was simply due to earlier AF onset, we examined the duration of paroxysmal AF (the time between the onset of AF and persistent AF). This duration was significantly shorter in choline-supplemented CREM-IbΔC-X mice compared with chow-fed or IMC-treated mice (**Figure 5B; Supplemental Table 9**). Collectively, these data suggested that TMAO contributes to both the onset and progression of AF, and that inhibition of the gut microbial TMAO pathway may represent a potential strategy to prevent AF development and progression.

Pharmacological targeting of gut microbial *CutC/D* resides in the gut and is not readily absorbed.

To evaluate the potential of IMC as pharmacological therapy, we first assed its absorption in mice receiving long-term IMC supplementation. Notably, the majority of IMC remained in the cecal contents of CREM-IbΔC-X mice, with minimal levels detected in plasma (**Figure 6A**). In addition, IMC treatment had no significant impact on hepatic FMO activity as monitored by direct examination of the metabolic transformation activity of TMA into TMAO in liver homogenates (**Figure 6B**). In contrast, mice chronically treated with IMC showed potent inhibition in cecal microbial enzymatic transformation capacity of d_9-choline into d_9-TMA under both aerobic and anerobic conditions (**Figure 6, C and D**). We observed that plasma TMAO concentrations in CREM-IbΔC-X mice fed supplemented with a choline diet and IMC were persistently suppressed for the duration of the mouse ECG study (approximately ~5 months) (**Supplemental Figure 14**). In contrast, CREM-IbΔC-X mice fed a choline diet alone exhibited persistently high plasma TMAO levels over the same period (**Supplemental Figure 14**). The persistent suppression or production of TMAO over time suggested that there were stable microbiota alterations present. These data also suggest that IMC effectively and chronically inhibits the TMA/TMAO gut microbial pathway, is not the result of off target effects on liver FMO activity, and could be considered for a long-term preventive therapeutic pharmacological agent.

Choline-supplemented diet altered gut microbiota composition in CREM-IbΔC-X transgenic mice and treatment with IMC reversed these alterations.

We next characterized the gut microbiota using metagenomic sequences analysis of cecum in a subset of male and female CREM-IbΔC-X mice used in the mouse ECG study. We observed that CREM-IbΔC-X choline supplemented mice used in the ECG study showed a significant decrease in gut microbiome diversity that was reversed with IMC treatment (**Figure 6E**). A canonical correspondence analysis plot demonstrated that mice on a choline diet showed differences in

diversity with microbial taxa compared to respective control groups (**Figure 6F**). Metagenomic associations are provided in **Supplemental Table 10 and 11**. Compositional differences of the gut microbiota showed that the relative abundance of the phylum Firmicutes was higher in CREM-IbΔC-X mice supplemented with a choline diet compared to chow and choline + IMC controls. (**Supplemental Figure 15A**, species highlighted in yellow). At the species level, the relative abundance of GGB29005_SGB41723 was greater in the choline supplemented diet compared to the chow and choline+IMC dietary control groups (**Supplemental Figure 15, A and B**). The species GGB18827_SGB41440 of the phylum Firmicutes was less abundant in choline group compared to the respective dietary control groups (**Supplemental Figure 15, A and B**).

CutC/D, catalytic subunit of choline TMA lyase, mRNA counts were significantly higher in choline diet compared to the chow and choline + IMC groups (**Figure 6G,** left panel). The same trend was observed in CREM-IbΔC-X mice after stratification by sex (**Figure 6G,** mid and right panel). We next investigated the association of genera that positively and inversely associated with the choline diet with the TMA, TMAO and *CutC/D* levels in CREM-IbΔC-X mice. A strong and significant positive correlation between the relative abundance of the gut microbial species GGB29005_SGB41723 and GGB18827_SGB41440 was observed between TMA, TMAO and C*utC/D* levels in CREM-IbΔC-X mice (**Figure 7A**). Although no significant inverse association was found between gut microbial species Lactobacillus_SGB5158 and GGB28980_SGB41692 with TMA and TMAO concentration, *CutC/D* levels showed a significant inverse relationship (**Figure 7A**). Other gut microbial species noted to have high or low relative abundances with CREM-IbΔC-X mice supplemented with a choline diet did not show a significant association with TMA, TMAO, or *CutC/D* (**Supplemental Figure 16**).

Gut microbial taxa associate with the onset of paroxysmal and persistent AF.

In final set of gut microbial studies, we assessed the changes in cecal microbial communities and the association with the time to onset of paroxysmal AF and persistent AF in CREM-IbΔC-X mice. Volcano plots (**Figure 7, B and C**) showed that several significant gut microbial species were associated with the time of onset of paroxysmal AF and persistent AF. In contrast, the duration of AF did not show any significant association with gut microbial species (**Supplemental Figure 17**). *Parvibacter caecicola* was the only species significantly associated with the time to paroxysmal AF. GGB30408_SGB43438 was significantly associated with the time to persistent AF. None of the gut microbial bacterial species had overlap with species that significantly associated with a choline diet.

A TMAO-promoting diet was associated with atrial remodeling in and CREM-IbΔC-X and C57BL/6J mice.

We next investigated whether dietary choline supplementation, with and without IMC, in CREM-IbΔC-X mice was associated with structural and functional changes in the atria and ventricles. Left atria (LA) size, measured in both diastole and systole, was significantly increased in male and female CREM-IbΔC-X mice fed a choline-supplemented diet compared with chow-fed controls (P<0.01) at both 5 weeks (**Supplemental Figure 18**) and 8 weeks of age (**Figures 8, A and C**). Notably, the LA size in both systole and diastole was significantly attenuated in the choline + IMC dietary group at 8 weeks of age (**Figures 8, A and C**), suggesting that pharmacological treatment with IMC can prevent TMAO-associated structural changes. In contrast, no significant differences were observed in left ventricular (LV) mass, LV ejection fraction (EF), cardiac output, fractional shortening, or heart rate among dietary groups, as measured by echocardiography at 5 weeks and 8 weeks of age (**Supplemental Figure 19-22)**. Since inflammation may play a role in atrial remodeling and AF, we also evaluated circulating inflammatory markers. Serum amyloid A (SAA) and interleukin-6 (IL-6) were not significantly different between choline-supplemented mice and chow-fed controls (**Supplemental Figure 23**).

We also performed echocardiograms on C57BL/6J male and female mice used in the transesophageal AF induction studies. Similar to CREM-IbΔC-X mice, C57BL/6J mice on a TMAO supplemented diet showed an increase in LA size that was significantly greater compared to chow C57BL/6J mice in both atrial diastole (P <0.01) and systole (P<0.01) (**Supplemental Figure 24, A and B**). Similar trends were observed when stratifying mice by sex (**Supplemental Figure 24A**)**.** In contrast, no significant differences in LV mass, LVEF, cardiac output, fractional shortening and heart rate, as measured by echocardiography among TMAO and chow groups in C57BL/6J mice at 6 weeks of age were observed (**Supplemental Figure 25**).

The NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome is not a significant contributor to the TMAO promoted AF.

We performed *in vitro* and *in vivo* studies examining the role of the NLRP3 inflammasome on the early onset of AF in choline supplemented CREM-IbΔC-X mice. We first exposed multiple cell types to physiological relevant levels of TMAO and examined the expression of *NLRP3* and *IL1β*. The cell types included human cardiac fibroblasts (**Supplemental Figures 26, A and B**), atrial engineered heart tissues (aEHTs) (**Supplemental Figures 27, A and B**), primary mouse atrial

and ventricular cardiomyocytes, and primary mouse atrial and ventricular fibroblast treated with vehicle or TMAO (**Supplemental Figures 28, 29**). We observed no significant increase in the relative mRNA expression of *NLRP3* or *IL1*β in cells exposed to TMAO. However, a significant decrease was detected in *IL1*β expression in primary atrial fibroblasts treated with TMAO at high concentrations (1 mM) compared with control (**Supplemental Figures 29**).

To investigate the role of the NLRP3 inflammasome in the early onset of atrial fibrillation observed in choline supplemented CREM-IbΔC-X mice, we performed mouse ECG experiments using a potent and selective inhibitor of NLRP3 inflammasome, MCC950. CREM-IbΔC-X mice supplemented with both choline and MCC950 in water had no significant change in developing paroxysmal AF compared to CREM-IbΔC-X mice supplemented with a choline diet (**Supplemental Figure 30A; Supplemental Table 12**). CREM-IbΔC-X mice supplemented with both choline in combination with MCC950 developed persistent AF significantly earlier compared to CREM-IbΔC-X mice supplemented with a choline diet alone (**Supplemental Figure 30B; Supplemental Table 13**). We confirmed that MCC950 was systemically inhibiting the NLRP3 inflammasome in CREM-IbΔC-X mice, by performing LPS IP injections and measuring plasma concentrations of IL-1β (a specific cytokine product of the NLRP3 inflammasome) and TNF-α, a nondependent NLRP3 cytokine. Compared to vehicle (PBS), there was no significant decrease in plasma concentrations of TNF-α, but a significant decrease in the concentration of IL-1β, consistent with MCC950 NLRP3 inhibition (**Supplemental Figures 30, C and D**). We also observed that the same CREM-IbΔC-X mice treated with choline in combination with MCC950 had higher TMAO levels compared to the chow diet fed control mice (**Supplemental Figure 30E**). Together, these data suggest that the NLRP3 inflammasome is not a significant pathogenic mechanism responsible in TMAO promoted AF in CREM-IbΔC-X mice.

A choline diet is associated with atrial electrophysiological dysfunction.

To understand the electrophysiological implications of a choline supplemented diet, we performed optical action potential mapping studies on female CREM-IbΔC-X mice (**Figure 9A**). Female CREM-IbΔC-X mice were selected due to relatively higher plasma TMAO levels as observed in this study and previous studies (35). Representative examples of atrial action potentials, activation isochrone maps, and local conduction velocity (CV) vectors are shown in **Figure 9B**. Measurement of average local CV vectors demonstrated a non-significant decrease in CREM-IbΔC-X female mice fed choline-supplemented diet compared to chow-fed controls diet (**Figure 9C**). In contrast, action potential duration at 80% repolarization APD80 and maximum APD80

were significantly reduced in the choline group, while minimum APD80 showed a trend toward significance (**Figure 9, D, E, and F**). Shortening of APD is a well-established mechanism underling increased susceptibility to AF (36-41). Consistent with this, the calculated cardiac wavelength (CV x APD80) was also significantly shorter in mice receiving choline supplementation (**Figure 9G**). Notably, reduced wavelength is associated with an increased vulnerability to AF (36-41).

In contrast, we found no significant change in beat rate, action potential duration at 50% repolarization (APD50), or calcium amplitude in aEHTs exposed to TMAO (**Supplemental Figure 31**). We also performed an *ex vivo* study of male and female CREM-IbΔC-X Langendorf perfused mouse hearts with direct TMAO exposure. Similarly, we found no significant changes in APD 50 and 80 or conduction velocity in optical mapping studies performed on *ex vivo* male and female CREM-IbΔC-X Langendorf perfused mouse hearts with direct TMAO exposure (**Supplemental Figures 32-34**).

TMAO promotes autonomic dysfunction by inhibition of the muscarinic receptor 2.

TMAO has structural similarities to acetylcholine. Optical mapping studies demonstrated that mice fed a choline-supplemented diet exhibited significantly shortened APD (**Figure 9D**). In addition, a prior report has shown that direct injection of TMAO into the ganglion plexi of the cardiac autonomic nervous system shortens the effective refractory period (ERP), a surrogate for APD duration, and increased AF inducibility in canine myocardium using a burst pacing protocol (32). Together, these data suggested that TMAO may promote AF through autonomic dysfunction, potentially via interaction with muscarinic receptors. To explore this mechanism, we generated a Human Embryonic Kidney 293 (HEK 293) cells stably expressing M_2 muscarinic receptor (M_2R) (**Figure 10, Supplemental Figure 35A-**Clone-1 was used for subsequent experiments), the most abundant muscarinic receptor subtype in the myocardium. In this system, TMAO acted as a significant inhibitor of M_2R signaling in the presence of the M_2R agonist, carbachol (**Figure 10A**). Furthermore, heart rate measurements in unanesthetized male and female CREM-IbΔC-X mice demonstrated that choline-supplemented mice had significantly higher heart rates, which were accompanied by elevated plasma TMAO levels (**Figure 10B and Supplemental Figure 35**). Collectively, these data support a role for TMAO in autonomic dysfunction and the promotion of AF.

Discussion

The present studies reveal both a clinical association between circulating levels of TMAO and AF risk, and through multiple animal model studies, a direct mechanistic link between elevated TMAO and AF onset and persistence that is reversed by pharmacological targeting of the gut microbial TMA/TMAO pathway. The promotion of AF by TMAO may be in part be mediated by autonomic dysfunction (**Figure 10C**). We first confirmed and extended previous clinical data linking TMAO to AF, by demonstrating that plasma TMAO, choline, and betaine independently associated with prevalent AF. Using a mouse model of spontaneous AF we next showed that a choline supplemented diet causes the CREM-IbΔC-X mouse to develop paroxysmal AF sooner (first time) AF and a persistent AF sooner than chow control mice. Plasma TMAO associated with the onset of both paroxysmal AF and persistent AF. This effect was completely abolished using the TMA lyase inhibitor, IMC. IMC is poorly absorbed into plasma and has no off-target effects on liver FMO activity. In contrast, IMC is highly abundant in the gut and effectively inhibited microbial TMA production from choline resulting in decreasing TMAO production and a delay in AF onset. In order to demonstrate that the effects of TMAO are not strain dependent, we validated that the direct supplementation of TMAO in the male and female C57BL/6J enhanced AF susceptibility. Characterization of the gut microbiome from CREM-IbΔC-X mice with a choline supplemented diet showed a significant increase in *CutC/D* mRNA counts and altered gut microbial species demonstrating that choline diet alters gut microbial TMA and its metabolite formation. Consumption of a choline or TMAO diet supplemented diet was significantly associated with atrial enlargement and atrial electromechanical dysfunction. This may in part be precipitated by chronic TMAO inhibition of the M_2R.

The TMAO pathway has been associated with the incidence of multiple CVD phenotypes in both animal models (2, 14, 15, 21, 42) and large clinical cohort studies (43-45). In two community-based cohorts, TMAO and its metabolites including carnitine and choline were associated with risk of heart failure (22). Similarly, this study extends the association of the TMAO pathway to the pathogenesis of AF in a large clinical biorepository. There are a few clinical studies reporting an association of increased circulating TMAO levels with increased AF risk. A study of two Norwegian cohorts with a long-term follow-up period of 7.3 years showed that a baseline elevated plasma TMAO concentration was associated with AF (HR: 1.16, 95% CI: 1.05, 1.28 and HR: 1.10, 95% CI: 1.004, 1.19) (35). In the recent AF-RISK study by Nguyen et al, it was observed that the elevated levels of TMAO associated with clinical AF progression (46). We add to these studies

by demonstrating that TMAO is independently associated with prevalent AF in a large clinical cohort at Cleveland Clinic. TMAO is a host–microbiome co-metabolite generated from dietary precursors such as choline, phosphatidylcholine, and L-carnitine through gut microbial metabolism, followed by hepatic oxidation via flavin-containing monooxygenase 3 (FMO3)(3, 12). Thus, inter-individual variability in TMAO levels may reflect differences in gut microbial composition and functional capacity, in addition to host factors such as liver enzyme activity and renal clearance. Although the absolute difference in TMAO concentrations between tertiles may not appear large, the associated risk for AF is substantial: there is a ~2 fold increase in the odds of developing AF. This is analogous to classic cardiovascular risk factors such as LDL cholesterol, where even modest elevations are associated with significant lifetime risk. These findings underscore that small differences in exposure over long periods can have large cumulative effects on disease risk. Despite these limitations, plasma TMAO remains an independent predictor of AF and other CVD phenotypes(2, 3, 21). We also demonstrate for the first time that choline and betaine also independently associate with prevalent AF in humans.

One of the most remarkable observations of this study was the demonstration that a choline supplemented diet was associated with the development of both earlier onset paroxysmal AF and persistent AF in the CREM-IbΔC-X mouse model of spontaneous AF. This effect was eliminated with the use of the gut microbial TMA lyase inhibitor IMC demonstrating that the gut microbiome is mechanistically involved in the pathogenesis of AF. After stratification by sex, the same trend was observed, however, in female mice, choline diet did not show a statistically significant difference compared to a chow diet for the onset of paroxysmal AF. This was also true of TMAO CREM-IbΔC-X mice supplemented with a TMAO diet. While CREM-IbΔC-X male and female mice supplemented with a choline diet had a similar onset of paroxysmal AF, chow CREM-IbΔC-X female appeared to develop paroxysmal AF sooner than male CREM-IbΔC-X mice. Notably, female CREM-IbΔC-X chow mice have higher circulating plasma TMAO levels than male CREM-IbΔC-X mice. This difference is related to CREM-IbΔC-X female mice producing more TMAO due to higher expression of hepatic FMO3 enzyme than male mice (9). Sex-dependent regulation of hepatic FMO3 expression is already well established. Prior studies have demonstrated that FMO3 expression in mice is markedly higher in females than males(12, 21), largely due to androgen-mediated repression and estrogen-associated upregulation(47, 48). Interestingly, CREM-IbΔC-X mice supplemented with IMC on a chow diet also showed a trend, and in the case of the paroxysmal AF (first time) in female CREM-IbΔC-X mice, a significant delay in the onset of AF. This is likely related to the presence of dietary choline in chow diet. What is notable, is that despite

the differences in TMAO plasma levels in male and female mice, we were able to see enhanced AF susceptibility in both sexes across two different mouse models of AF possible suggesting there is a threshold effect of TMAO inducing AF.

Prevailing evidence suggests the need for future research to study the dietary and pharmacological inhibition of gut-microbial metabolites like TMAO formation as a potential treatment strategy for attenuating the development of AF. This study is the first to demonstrate the use of TMA lyase inhibitors as pharmacologic agents for the treatment of AF. In addition to mouse ECG studies suggesting using IMC to prevent AF and mouse studies showing IMC reverses TMAO induced atrial remodeling, we also demonstrate that the vast majority of IMC remains in the gut and relatively little circulates in mouse plasma. Moreover, the unaltered conversion of d_9-TMA to d_9-TMAO in the liver suggests there are no further effects of IMC on the oxidation of TMAO by FMOs in the liver that could account for lower overall circulating TMAO concentrations. These animal studies support the use of TMA lyase inhibitors in human studies.
Our characterization of the gut microbiome showed that CREM-IbΔC-X mice supplemented with a choline diet had significantly less diversity and an increased abundance of Phylum Firmicutes (GGB29005_SGB41723 and GGB18827_SGB41440) when compared to control groups. Not surprisingly, we observed that relative abundance of both the species of the phylum Firmicutes was strongly and positively correlated with the TMA, TMAO and *CutC/* D levels in CREM-IbΔC-X mice supplemented with different diets. Our study findings are in line with the previous studies which found that IMC treatment alters the gut microbiome in favor to the host (5, 15, 49-52). It was observed that the ratio of Firmicutes to Bacteroidetes which has been linked to obesity in human and mice was significantly suppressed by IMC treatment in mice fed with high fat diet (49-52). In a study by Schugar et al., it was observed that IMC significantly reduced the proportions of some microbial taxa including Clostridiales that belong to the phylum Firmicutes and is significantly associated with the plasma TMA and body weight in a mice model (5). In the current study, the significant positive correlation of the two species GGB29005_SGB41723 and GGB18827_SGB41440 from the phylum Firmicutes with the plasma levels of TMA, TMAO and *CutC/D* levels may explain the gut microbial alterations with IMC treatment. Interestingly, none of the gut microbial bacterial species associating with the choline diet, TMA, TMAO or *CutC/D* had an association with the time of onset of paroxysmal AF or persistent AF. The association of *Parvibacter caecicola* and GGB30408_SGB43438 with the onset of paroxysmal AF and persistent AF, warrants further study.

In our study, IMC effectively inhibits TMAO without off-target liver effects and its long-term impact on the gut microbiota was stable for 5 months. However, AF recurrence following IMC cessation was not studied as the CREM-IbΔC-X mouse model is a progressive mouse model of AF leading to permanent AF. Once established, it cannot be reversed due to significant atrial myopathy (53, 54). The mouse model follows a similar pathway of the irreversible nature of human persistent and permanent AF. Although microbiota perturbations have been associated with AF phenotypes, including structural and electrophysiological remodeling, causal and reversible microbiota interventions in AF remain an area for future study.

This study suggests that TMAO is mechanistically involved in the onset and progression of AF. While plasma concentration of TMAO was higher in CREM-IbΔC-X supplemented with a choline diet and predicted the onset of both paroxysmal AF and persistent AF, levels of betaine and choline did not reliably predict the onset of paroxysmal AF or persistent AF. This observation suggests a crucial role for TMAO in the progression of AF. To further assess the role of TMAO in the pathogenesis of AF, we provided a provisionally supplemented CREM-IbΔC-X mice with a TMAO diet and demonstrate that, similar to CREM-IbΔC-X mice supplemented with choline, TMAO causes mice to develop paroxysmal AF and persistent AF sooner compared to chow diet. This unambiguously demonstrates that TMAO is directly involved in the pathogenesis of AF.

We demonstrate that CREM-IbΔC-X-TG female mice supplemented with a choline diet had a shorter cardiac wavelength compared to controls. A shortened wavelength is known to be associated with an increased vulnerability to AF and is a mechanism of reentrant excitation (36-41). Although there was not a statically significant decrease in conduction velocity in choline supplemented mice, a shortened APD (equivalent to effective refractory period) has been implicated in AF in mice supplemented with a high fat diet (55) and in increased vulnerability of both AF and ventricular tachycardia in humans (38, 41). A longer path length (a larger atrial area) likely from LA dilation is part of the wavelength hypothesis and can also contribute to an increase in stability (e.g. persistent AF) and vulnerability to AF (40). Thus, a combination of both structural and electrophysiological dysfunction is likely contributing to AF in choline supplemented mice.

Overall, systemic inflammation does appear to be a major mechanism in AF promoted by TMAO. Studies show that inflammatory markers like C-reactive protein and interleukin (IL)-6, are positively correlated with LA diameter, suggesting that inflammation may play a role in atrial remodeling and prolong the duration of AF (56). In our study, we did not find any association of a

choline diet with systemic SAA protein in mice, an acute-phase inflammatory protein similar to CRP or IL-6 in humans. TMAO has been implicated in the priming and activation of the NLRP3 inflammasome in studies examining vascular inflammation and dysfunction (57) And work lead by Li and colleagues has demonstrated there is enhanced activation of NLRP3-inflammasome in patients with paroxysmal AF and persistent AF and that the NLRP3 inflammasome is critical in the pathogenesis of AF in CREM-IbΔC-X mice (58). This suggested a possible link between TMAO, the NLRP3 inflammasome, and AF. We performed comprehensive *in vivo* and *in vitro* studies that demonstrate that the mechanism of TMAO enhanced AF does not appear to be though the NLRP3 inflammasome in CREM-IbΔC-X mice. These observations suggest that TMAO and the priming/activation of the NLRP3 inflammasome is not a universal pathogenic mechanism in cardiovascular diseases.

A possible pathogenic culprit for the development of atrial myopathy and electromechanical dysfunction is chronic autonomic dysfunction from TMAO inhibition of M_2R and increasing sympathetic tone. Classically, M_2R agonists acutely shorten action potential duration and increase AF susceptibility. In contrast, M_2R acute inhibition prolongs APD and decreases AF susceptibility. However, the long-term effects of M_2R inhibition are not as well understood. Clinically, inhaled long-acting muscarinic receptor antagonists (LAMA) are associated with worse composite cardiovascular outcomes. This included cardiac arrhythmias and in a subgroup analysis there was significant increase arrhythmogenic events amongst subjects taking LAMAs (59). Another study of antipsychotic drugs, well known to have anticholinergic side effects, first found that subjects that were concomitantly taking anticholinergic mediations had an increase in incident AF. Additionally, subjects that chronically took antipsychotics drugs were found to have an increase in AF incidence. This association was found to correlate with antipsychotic drugs with increasing binding affinity to muscarinic receptors. This effect was most notable with antipsychotic drugs that had a strong affinity for M2 receptor blockade where a 22% increase in AF incidence was noted (60). The pathogenic mechanism responsible for more AF is hypothesized to be a relative increase in sympathetic tone relative to the inhibited parasympathetic pathway. Consistent with autonomic imbalance and increase in sympathetic tone, is the observation that unanesthetized CREM-IbΔC-X mice supplemented with a choline diet had significantly elevated heart rates.

In classic basic work completed by Patterson et al. a complex autonomic interplay between parasympathetic and sympathetic activity demonstrating overall that shortening of the APD was associated with triggered firing from the canine pulmonary veins and an increase in AF

susceptibility (61). Additionally, increases in sympathetic tone is well known to shorten APD and increase susceptibility to atrial fibrillation (62, 63). Together these data suggest a role of TMAO in autonomic dysfunction via inhibition of the M_2R and resulting an increase in sympathetic tone and susceptibility to AF. Additionally, increases in sympathetic tone is well known to shorten APD and increase susceptibility to atrial fibrillation (62, 63). Short term exposure of TMAO to *ex vivo* CREM-IbΔC-X mouse hearts or aEHTs did not demonstrate electromechanical dysfunction. This suggests that TMAO involvement in the pathogenesis of AF may require a longer term TMAO exposure to cardiomyocytes, could produce its effects via other cell types found in the heart, or produce its proarrhythmogenic effects via extracardiac mechanisms. Together these data suggest a role of TMAO in autonomic dysfunction via inhibition of the M_2R and resulting increasing sympathetic tone and susceptibility to AF.

Conclusions.

TMAO promotes AF in a gut microbiota dependent manner by causing autonomic dysfunction, atrial myopathy, and electromechanical dysfunction. TMAO promoted AF is inhibited by dietary supplementation with IMC. These data represent a novel link between the gut microbiome and AF and suggests that pharmacological inhibition of the choline catabolic pathway may be a preventive treatment for AF.

METHODS

Detailed methods can be found in supplemental methods.

Sex as a biological variable.

In human participants, sex was considered as a biological as demonstrated in the subgroup analysis in **Figure 2**. In mouse studies all findings are reported for both sexes except for optical mapping studies. Female CREM-IbΔC-X mice were selected due to relatively higher plasma TMAO levels compared to male mice (21).

Cardiovascular research subjects.

Plasma TMAO, choline, and betaine concentrations were quantified in subjects by stable isotope dilution liquid chromatography with on-line tandem electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) as previously described (42). These investigations used archival plasma specimens (N=5090) from the GeneBank of Cleveland Clinic cohort (GeneBank: NCT00590200 https://clinicaltrials.gov/ct2/show/NCT00590200), a research tissue

repository comprised of sequential consenting stable subjects undergoing elective cardiac evaluation with connecting clinical longitudinal outcome data (2, 3, 64, 65). Exclusion criteria for GeneBank included patients with a recent myocardial infarction (<4 weeks) or elevated troponin I (>0.03 mg dl^{-1}) at enrollment. Quantitative TMAO, choline and betaine data were available for all (N=5090) subjects. CVD was clinically defined as having a previous history of documented coronary artery disease, peripheral artery disease, cerebral vascular disease (history of a transient ischemic attack or cerebrovascular accident), history of revascularization (coronary artery bypass graft, angioplasty, or stent) or significant angiographic evidence of coronary artery disease (≥50% stenosis) in at least one major coronary artery at the time of coronary angiography. History of AF, heart failure, myocardial infarction, hypertension, and diabetes were adjudicated by medical personnel at the time of subject enrollment into Genebank.

Experimental animals.

Characteristics of cardiomyocyte-directed overexpression of cAMP response element modulator (CREM-IbΔC-X) mice were described previously (33, 34). CREM-IbΔC-X hemizygous mice were used in experimentation and bred by crossing a hemizygous CREM-IbΔC-X mouse with a (FVB/NJ - JAX-001800). WT (C57BL/6J mice - JAX-000664) using needle electrode electrocardiograms (ECG), and transesophageal pacing technique, respectively (66), as previously described. More detailed experimental groups of these studies can be found in **Supplemental Methods**.

Mouse model of AF and mouse ECG.

Cardiac rhythm was assessed in hemizygous CREM-IbΔC-X mice and WT (C57BL/6J mice - JAX -000664) using needle electrode electrocardiograms (ECG), and transesophageal pacing technique as previously described (54, 67). More details on the ECG recordings can be found in **Supplemental Methods**.

Shotgun metagenomics sequencing and bioinformatics analysis.

Gut microbial sequencing was performed using cecum samples from CREM-IbΔC-X mice on chow, choline, chow+IMC and choline+IMC diets. Quality assessment of metagenomic sequences was performed following established protocols (68). Additional details on the metagenomics sequencing and analysis can be found in **Supplemental Methods** .

Quantification of TMAO, TMA, Choline, Betaine, and IMC.

Stable isotope dilution Liquid Chromatography-Tandem Mass Spectrometry(LC-MS/MS) on a Shimadzu 8050 triple quadrupole mass spectrometer was used to quantify TMAO, TMA, choline, betaine and IMC from acidified mouse plasma samples using electrospray ionization in positive ion mode with multiple reaction monitoring, as previously described (2, 42, 69).

Creation and maintenance of EHTs.

We created engineered heart tissues (EHT) from dissociated atrial-like iCMs suspended in a fibrin hydrogel between flexible silicon posts (EHT Technologies DiNAQOR, Hamburg, Germany) (70). More details on the preparation and maintenance of EHTs can be found in **Supplemental Methods** .

Statistical analysis.

Baseline characteristics, demographics, and laboratory values of Genebank subjects with and without a history of AF were compared using a Wilcoxon rank-sum test for continuous variables and a Pearson's Chi Square test for categorical variables. Pearson's Chi Square test was also used to assess the relationship between the proportion of prevalent AF and tertiles of increasing plasma concentrations of TMAO, choline, and betaine. Odds ratio of prevalent AF and tertiles of increasing concentrations of plasma TMAO, choline, and betaine with no adjustment, after adjustment for patient characteristics and comorbidities (Model 1: age, sex, diabetes mellitus (DM), cardiovascular disease (CVD), systolic blood pressure (SBP), current smoker, body mass index (BMI) and high sensitivity C reactive protein (hsCRP) and full model adjustment was performed (Model 2: Model 1+ estimated glomerular filtration rate (eGFR)). Cubic spline plots were performed using an unadjusted logistic regression analysis of increasing plasma choline, betaine, and TMAO plasma concentrations. In the cubic spline plots, associations were modeled using restricted cubic splines with 3 knots in a logistic regression model, yielding 2 spline degrees of freedom (one for the linear term and one for nonlinearity). Knot locations were placed at the default quantiles for 3 knots (approximately the 10th, 50th, and 90th percentiles of the metabolite distribution). P-interaction values were obtained from likelihood ratio tests comparing logistic

models with and without the interaction between TMAO tertiles and each subgroup factor. Kaplan–Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratio (HR) and 95% confidence intervals (CI). Multiple groups were compared with a Kruskal-Wallis test with a Dunn post hoc analysis to determine the significant difference between the medians of multiple independent diet groups and multiple paired comparison adjustments were performed. The Mann-Whitney test was used to compare the two medians of two independent groups. Permanova was used to compare the b diversity distributions of different dietary groups. Student's t test was used to compare the means of CV, APD and wavelength between two diet groups after testing for the normality and lognormality tests of shapiro-Wilks test and Kolmogorov-Smirnov test. Benjamini–Hochberg false discovery rate correction to adjust q-values for multiple testing was performed to assess the gut microbial abundance and its association with the onset of paroxysmal AF, persistent AF, and duration of AF. The statistical significance level was set at $P < 0.05$. All data was analyzed using R software version 4.3.3; R Core Team, 2024 and Prism (GraphPad Software).

Study Approval:

CREM-IbΔC-X mice were obtained from the laboratory of Na Li, PhD (Baylor College of Medicine) with permission from Frank U Müeller. Breeders of all WT (C57BL/6J mice) mice were obtained from Jackson Laboratories. All animal experiments were approved by the Cleveland Clinic IACUC. All human participants from GeneBank gave written informed consent and the Institutional Review Board of the Cleveland Clinic approved the study protocol.

Data Availability:

The data supporting the findings of this study are available within the article and in Supplemental Tables 1-13 and Figures 1-35. Values for all data points in graphs are reported in the Supporting Data Values file. The next-generation sequencing data generated in this study have been deposited in a MINSEQE-compliant public database and are available under the NCBI BioProject accession number PRJNA1482443 (URL:https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1482443). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Author contributions

S.A. conducted the experiments, analyzed the data, and wrote the manuscript X.S.L , N.S., K.R.L. conducted experiments, helped design the experiments, analyzed the data, and revised the manuscript. I.P.,L.A., E.O., D.V., I.N., H.S.K., H.M., Z.W., J.A.L., M.Y.T., K.M., D.P.M., S.S., J.H.R., and V.C. helped conduct the experiments, analyzed the data, and revised the manuscript. D.R.V.W., J.B, M.K.C, J.D.S., O.W., and S.L.H. helped design the experiments and revise the manuscript. R.A.K. conceived the project, conducted the experiments, analyzed the data, and designed the experiments, and wrote manuscript.

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Conflict of Interest Statement

Z.W. and S.L.H. are named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics and have the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab, a fully owned subsidiary of Quest Diagnostics and Zehna Therapeutics. S.L.H. also reports having been paid as a consultant from Zehna Therapeutics and has received research funds from Zehna Therapeutics.

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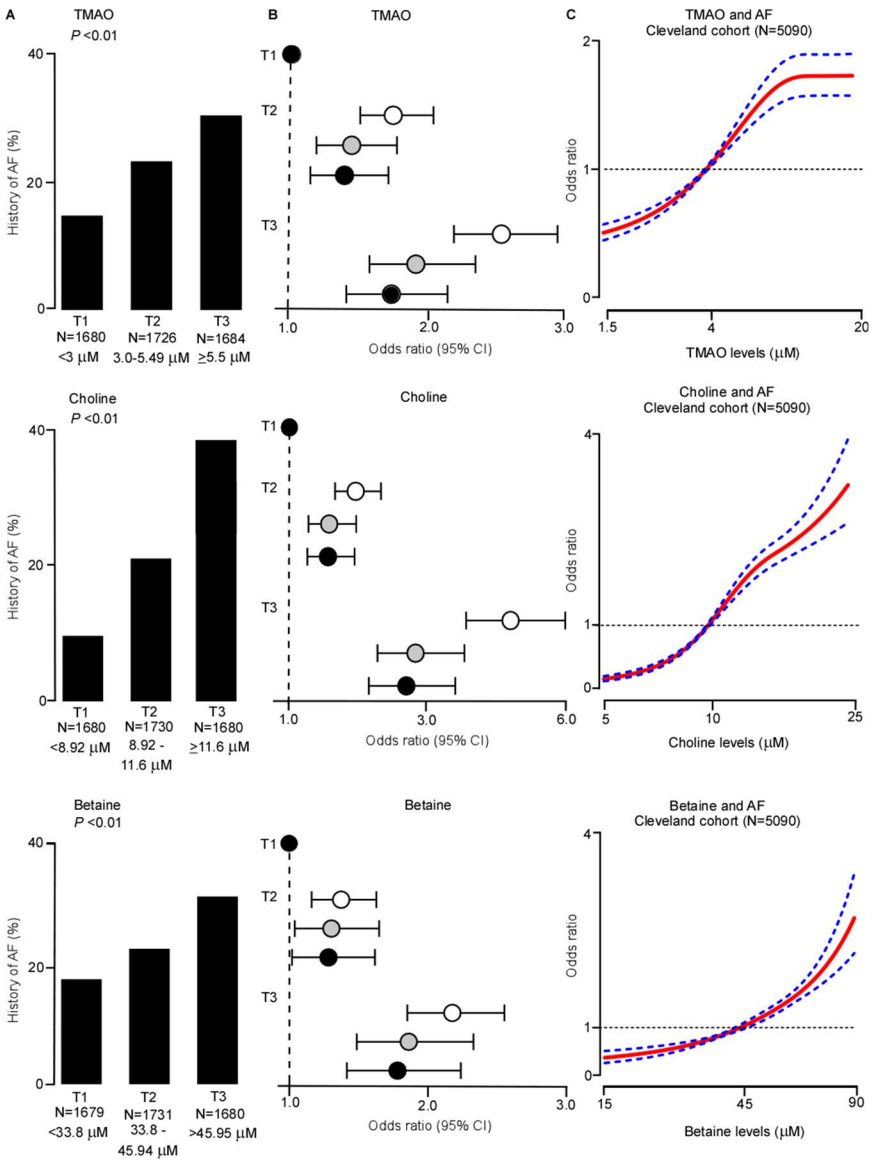

Figure 1: Plasma TMAO, choline, and betaine independently associate with prevalent AF. (**A**) The relationship between tertiles (T) of increasing plasma concentrations of TMAO, choline, and betaine and the proportion of prevalent AF in Genebank subjects. The *P* value represents a Pearson's Chi Square test. (**B**) Forest plots of the odds ratio of prevalent AF and tertiles of increasing concentrations of plasma TMAO, choline, and betaine with no adjustment (open circles), adjustment for patient characteristics and comorbidities (model 1: age, sex, DM, CVD, SBP, current smoker, BMI and hsCRP; gray circles), and after full model adjustment (model 1 and eGFR; black circles). The circles represent the odds ratio and the horizontal bars extend from the lower limit to the upper limit of the 95% CI. (**C**) Cubic spline plots of plasma concentrations of TMAO, choline, and betaine and the odds ratio of prevalent AF. The solid red line represents the estimated OR from unadjusted logistic regression analysis at increasing values of the metabolite and the dashed blue lines represent the 95% CI. Abbreviations: AF, atrial fibrillation; BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; CAD, coronary artery disease; DM, diabetes mellitus; eGFR, estimated glomerular fraction rate; hsCRP, high sensitivity C-reactive protein; SBP, systolic blood pressure; T, tertile; TMAO, trimethylamine *N*-oxide. ***P* < 0.01.

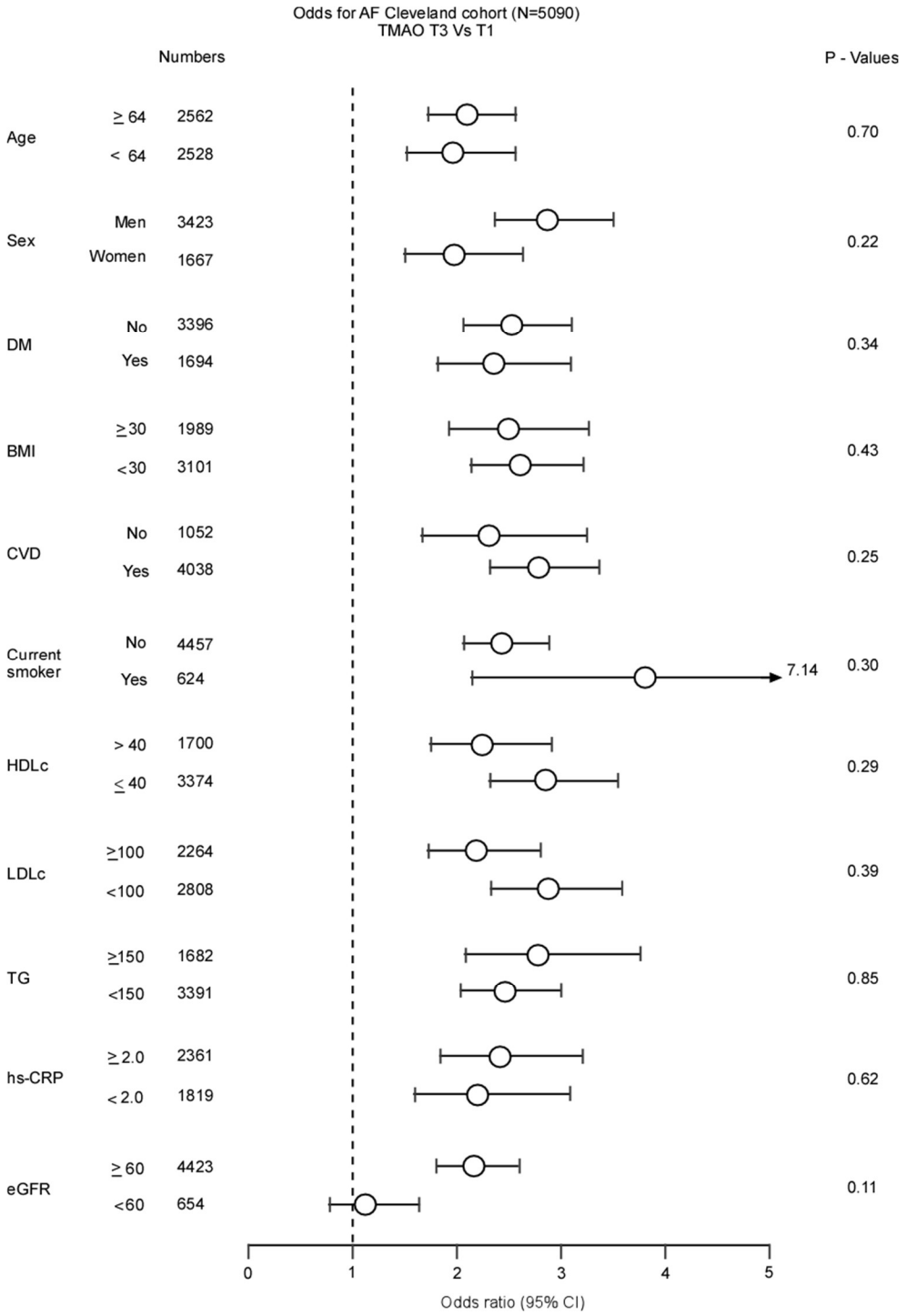

Figure 2: Association of baseline TMAO tertiles with odds of AF. Forest plots show odds ratio for AF with increasing TMAO levels in the indicated subgroups. Open circles represent odds ratio for tertile 3 (T3) versus tertile 1 (T1); horizontal lines indicate 95% confidence intervals. *P*-interaction (*P*) values were obtained from likelihood ratio tests comparing logistic models with and without the interaction between TMAO tertiles and each subgroup factor. Abbreviations: AF, atrial fibrillation; BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; CVD, cardiovascular disease; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; TG, triglycerides. Units for continuous variables: age (years), BMI (kg/m²), HDLc (mg/dL), LDLc (mg/dL), TG (mg/dL), hs-CRP (mg/L), eGFR (mL/min/1.73 m²).

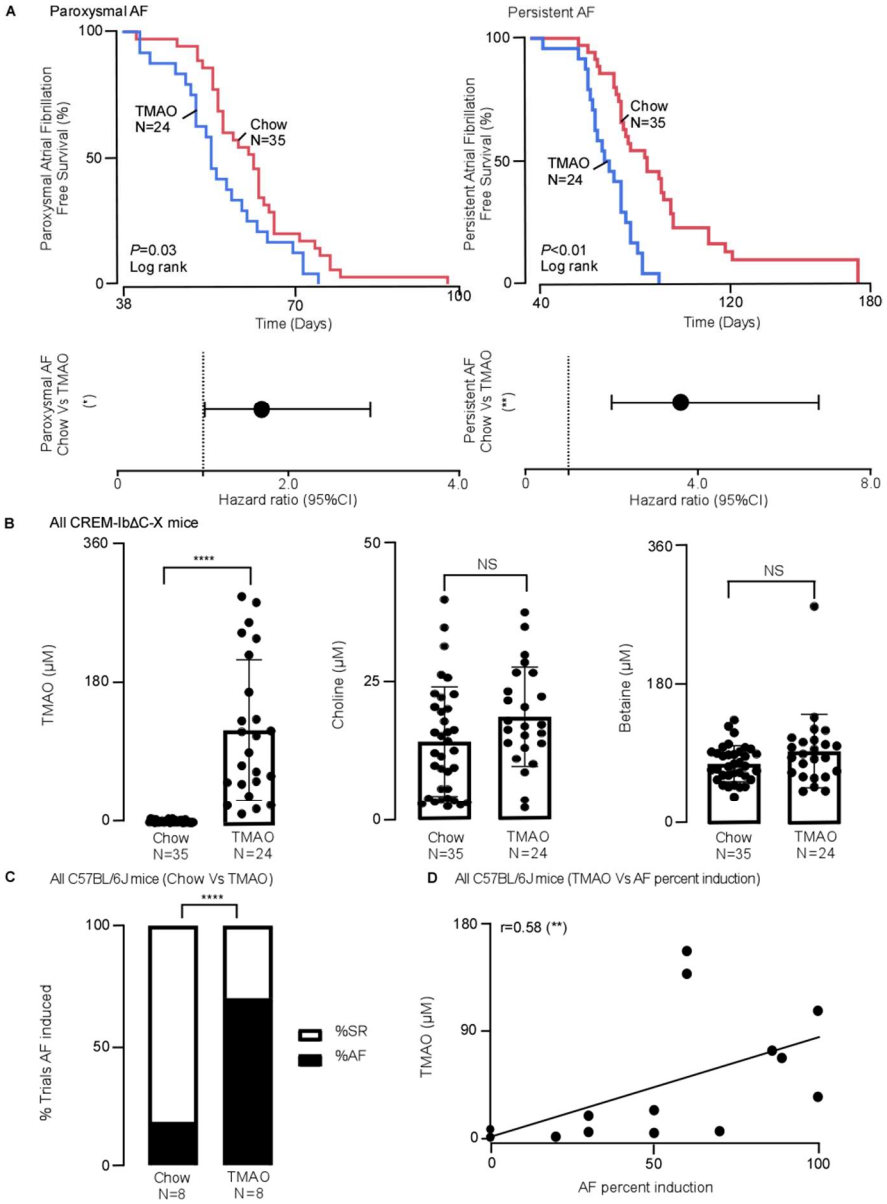

Figure 3: Kaplan Meier plots and hazard ratios of the incidence of AF in CREM-IbΔC-X mice on TMAO diet and plasma TMAs concentrations. (**A**) The incidence of paroxysmal and persistent AF in all CREM-IbΔC-X mice represented by a Kaplan Meier Plot and hazard ratios (HR) on chow diet or a TMAO supplemented diet. The circles represent the HR and the horizontal bars extend from the lower limit to the upper limit of the 95% CI of the estimate of the HR. HR between respective dietary groups were calculated using a Cox proportional hazard model. (**B**) Plasma concentrations of TMAs in all CREM-IbΔC-X mice used in the TMAO mouse ECG study. A Mann Whitney test was used to assess significant differences between groups. Each box plot represents mean ± SD. (**C**) Trials of AF percent induction in C57BL/6J mice treated with TMAO and chow diets. (**D**) TMAO levels and AF percent induction correlation in all C57BL/6J mice. A Pearson correlation coefficient measuring the relationship between TMAO levels and AF percentage of AF. *P* value determined using the linear regression model. AF, atrial fibrillation; CI, confidence interval; TMAO, trimethylamine *N*-oxide. ***P* < 0.01, *****P* < .0001, NS, Non-Significant.

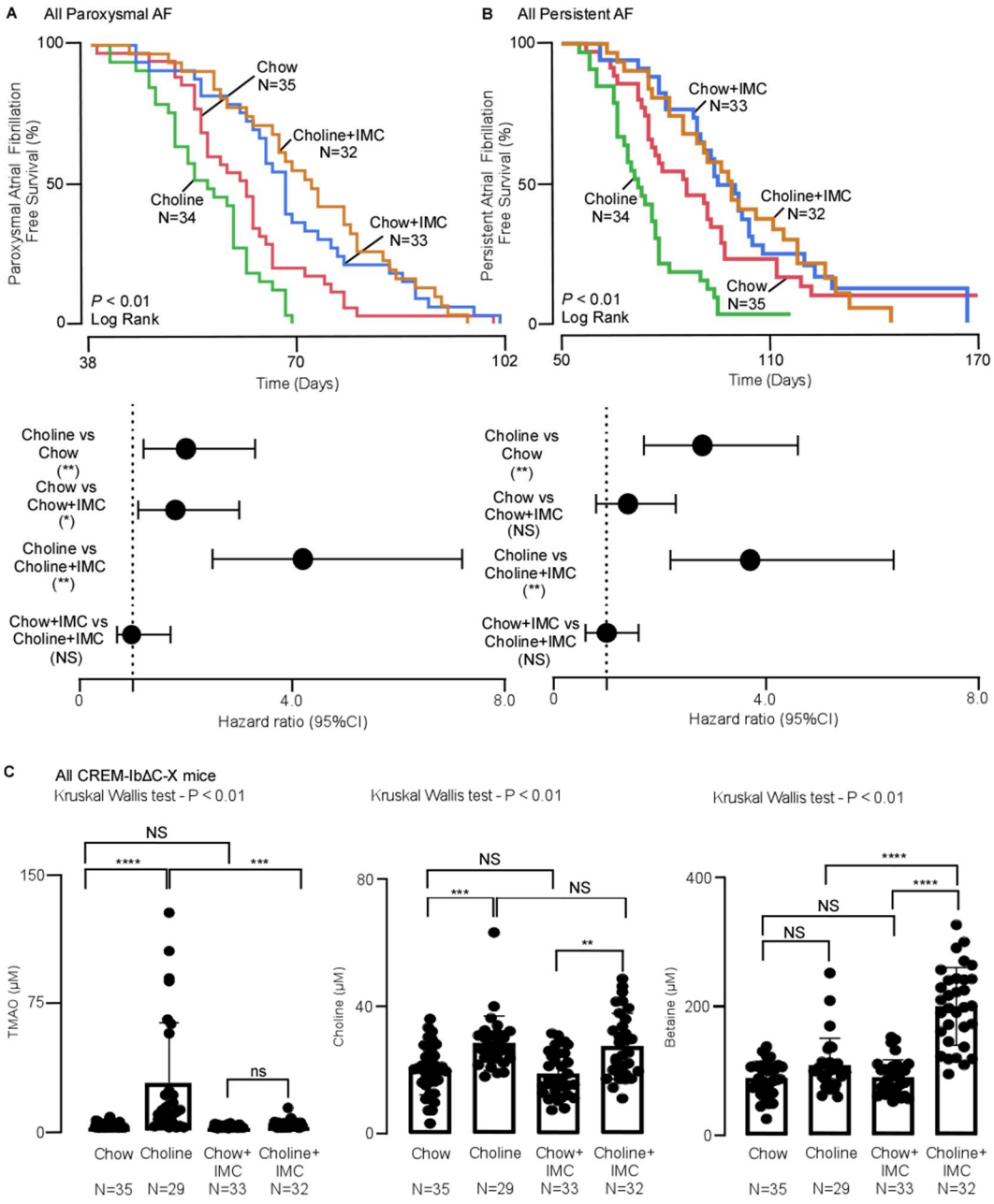

Figure 4: Kaplan Meier plots and hazard ratios of the incidence of paroxysmal AF and persistent AF in CREM-IbΔC-X mice on respective diets. The incidence of paroxysmal AF in all (**A**), persistent AF (**B**) in all CREM-IbΔC-X mice represented by a Kaplan Meier Plot (top panel) and Hazard ratios (HR) (bottom panel) on chow diet, choline supplemented diet, a chow + IMC supplemented diet, and a choline + IMC supplemented diet. The circles represent the HR and the horizontal bars extend from the lower limit to the upper limit of the 95% CI of the estimate of the HR. HR between respective dietary groups were calculated using a Cox proportional hazard model. (**C**) Average plasma concentrations of TMAO, choline and betaine in all CREM-IbΔC-X mice used in ECG study. Each box plot represents mean ± SD. A Kruskal-Wallis test with Dunn's *post hoc* analysis was used to determine the statistically significant difference between the medians of four independent diet groups and multiple paired comparison adjustments were performed. NS, Non-Significant, **P* <0.05, ***P* < 0.01, ****P* < 0.001, *****P* <0 .0001. AF, atrial fibrillation; CI, confidence interval; IMC, idomethylcholine; TMAO, trimethylamine *N*-oxide.

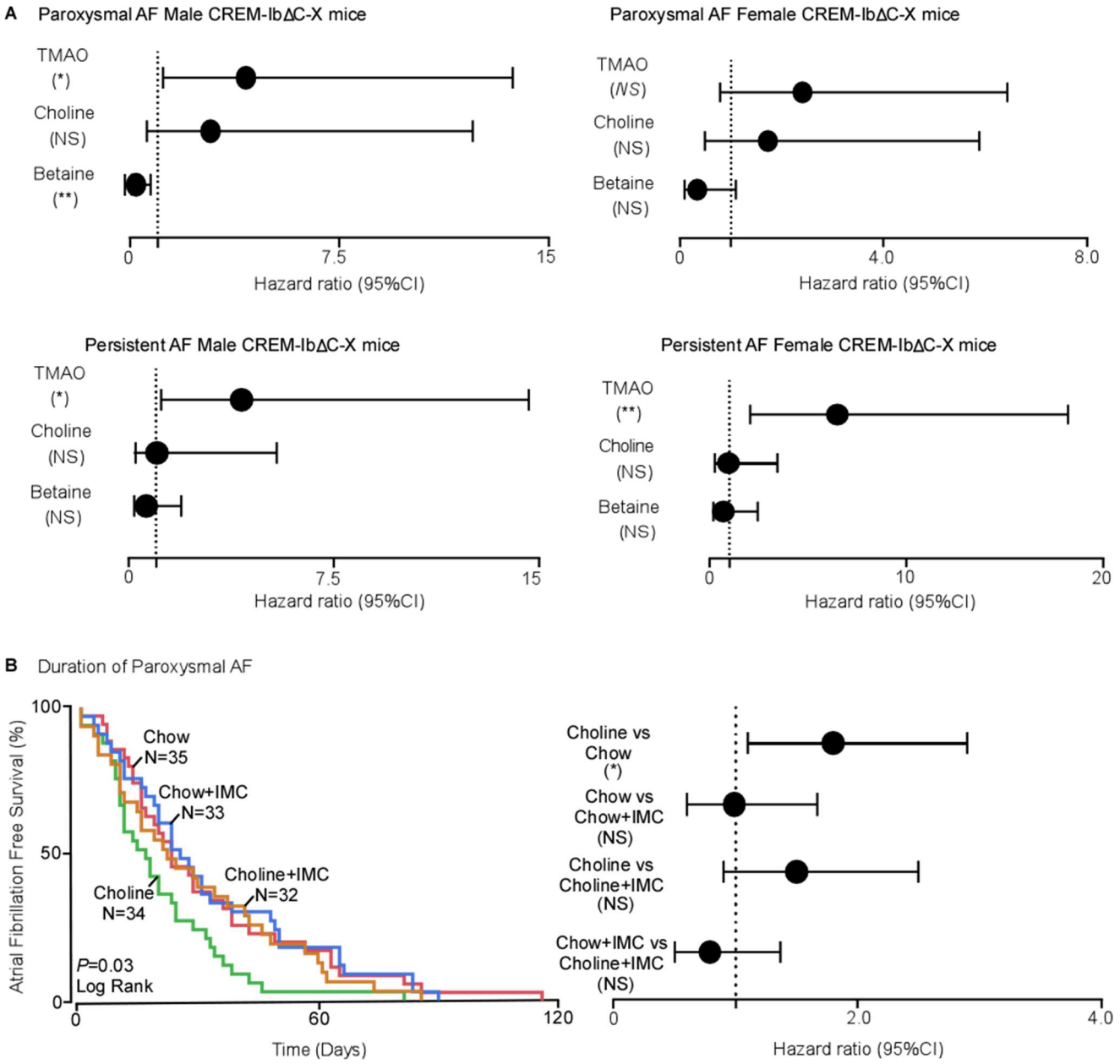

Figure 5: Hazard ratios and Kaplan Meier plots of the incidence of paroxysmal AF and persistent AF. (**A**) The incidence of paroxysmal AF and persistent AF and average plasma TMAO, Choline, Betaine concentrations in both male and female CREM-IbΔC-X mice. (**B**) Kaplan Meier plots (top pamel) and Hazard ratios (HRs) (bottom panel) of the duration of paroxysmal AF in male and female CREM-IbΔC-X mice. In A-B, the circles represent the HR and the horizontal bars extend from the lower limit to the upper limit of the 95% CI of the estimate of the HR. HR between respective dietary groups were calculated using a Cox proportional hazard model. Each HR represents the risk over the entire analyte concentration range. AF, atrial fibrillation; IMC, idomethylcholine; TMAO, trimethylamine *N*-oxide. NS, Non-Significant; **P* <0.05, ***P* < 0.01.

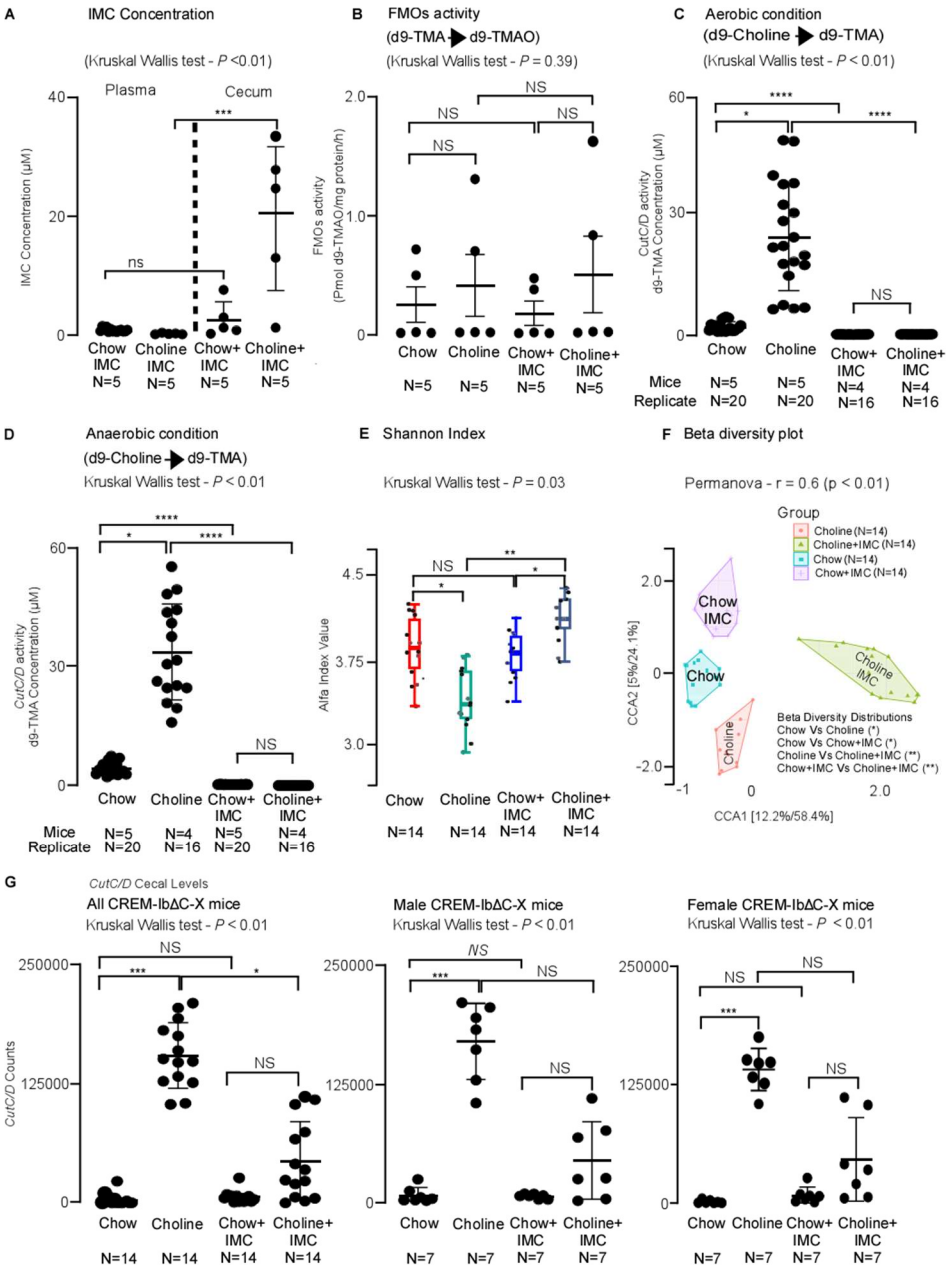

Figure 6: IMC concentrations in plasma and cecum samples and assessment of *CutC/D* activity. (**A**) Quantification of IMC in cecum and plasma samples of CREM-IbΔC-X mice on respective diets. (**B**) Quantification of the conversion of d_9-TMA to d_9-TMAO in liver samples of mice on respective diets. (**C**) Quantification of the conversion of d_9-choline to d_9-TMA in cecum of CREM-IbΔC-X mice under aerobic and (**D**) Anaerobic condition on respective diets. Replicates which represent 4 independent samples from each mouse. (**E**) α-Diversity plot (Shannon index) and (**F**) Principal component analysis of β-diversity plot of CREM-IbΔC-X mice on respective diets. Permanova was used to compare the beta diversity distributions of dietary groups. (**G**) *CutC/D* mRNA counts in cecum samples from all, male, and female CREM-IbΔC-X mice utilized in the mouse ECG study. In **A-E** and **G**, each line denotes mean ± SD. A Kruskal-Wallis test with Dunn's *post hoc* analysis was used to determine the statistically significant difference between the medians of four independent diet groups and multiple paired comparison adjustments were performed. CCA, canonical correspondence analysis; ECG, electrocardiogram; IMC, idomethylcholine; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; NS, Non-Significant; **P* <0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

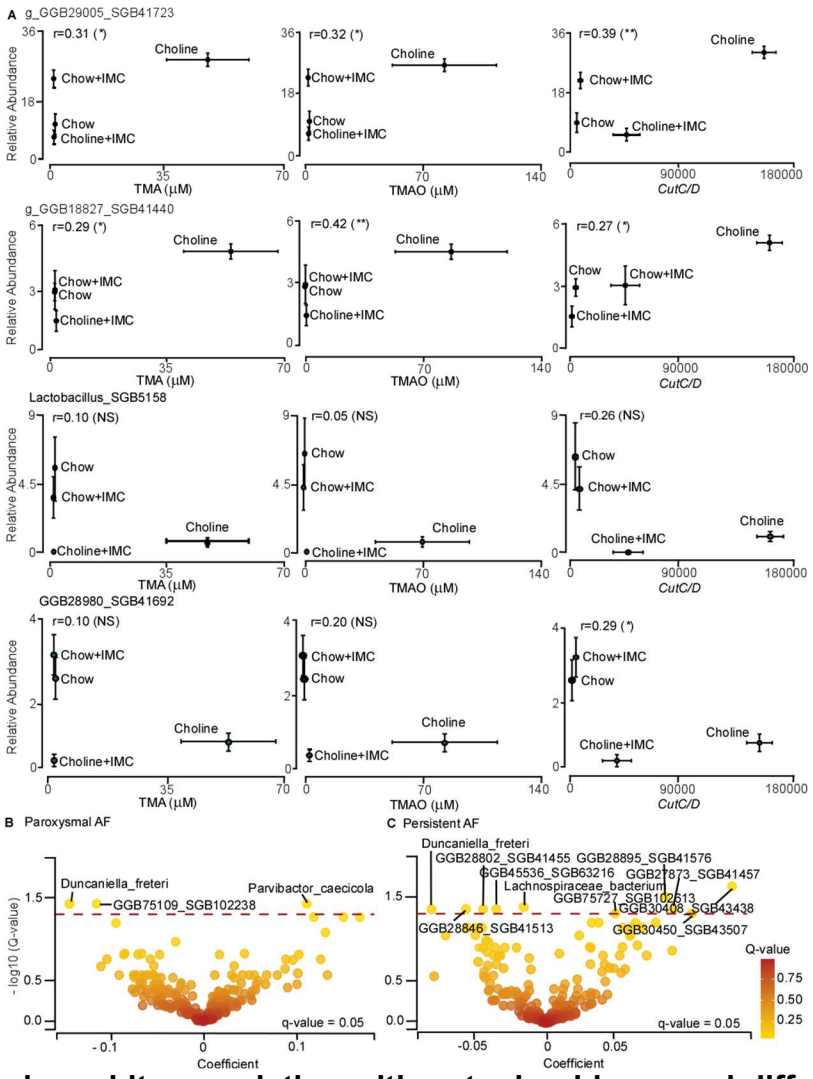

Figure 7: *CutC* levels and its correlation with gut microbiome and differential abundance of gut microbial species with the time of onset of paroxysmal AF and persistent AF (**A**) Gut microbiota taxa abundance and its association with mouse plasma TMA and TMAO and *CutC/D* mRNA counts in linked cecal samples of male and female CREM-IbΔC-X mice on respective diets. The circles represent the mean, and the bars represent the standard errors with regression and P values determined using the linear regression model. NS, Non-Significant, **P* <0.05, ***P* < 0.01, ****P* < 0.001. TMA, trimethylamine; TMAO, trimethylamine *N*-oxide. Volcano plot showing the association of differential abundance of gut microbial species with the time of onset of (**B**) paroxysmal AF and (**C**) persistent AF. In the plot, the x-axis represents the coefficient (effect size and direction) and the y-axis showing the -$\log_{10}$(Q-value). Each circle represents a specific microbial species, colored on a yellow-to-red gradient indicating q-values, with yellow being more significant. The dashed dark red line at y≈1.30 marks the statistical significance threshold (q-value=0.05). Circles above this line and labeled are statistically significant (q<0.05). Circles in the upper corners of the plot, far from zero on the x-axis and high on the y-axis, represent features with both large effect sizes and high statistical significance. The Benjamini–Hochberg false discovery rate correction was used to adjust q-values for multiple testing.

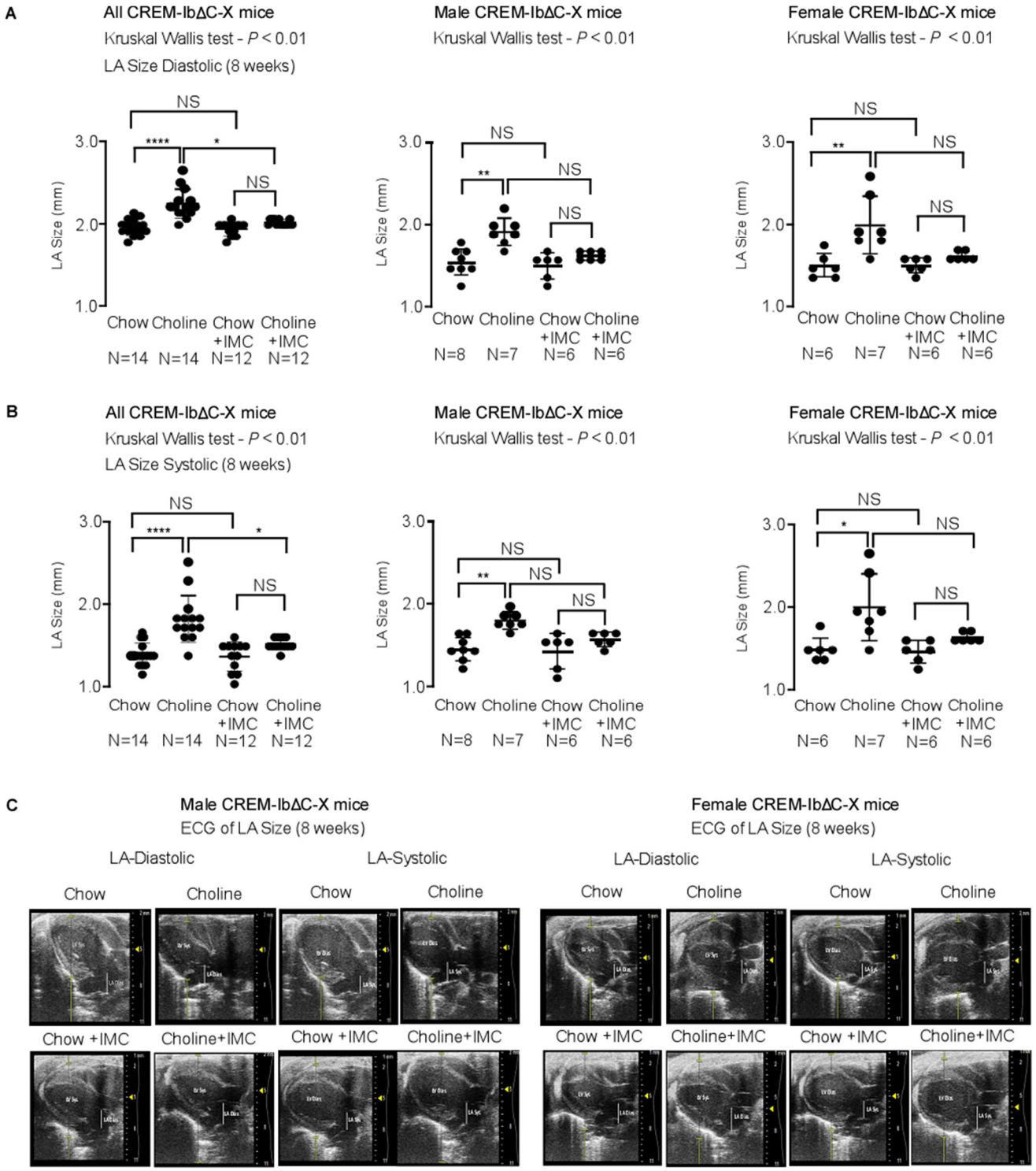

Figure 8: Choline diet increases the left atrium (LA) size during the diastolic and systolic function. (**A**) LA diastolic in size, male and female CREM-IbΔC-X mice at 8 weeks of age on respective diets. (**B**) LA systolic size in male and female CREM-IbΔC-X mice at 8 weeks of age on respective diets. (**C**) Echocardiography (ECHO) of LA size in female and male CREM-IbΔC-X mice at 8 weeks of age at respective diets. In A-B, the line denotes mean ± SD. A Kruskal-Wallis test with a Dunn's post hoc analysis was used to determine the significant difference between the medians of four independent diet groups and multiple paired comparison adjustments were performed. NS, Non-Significant, *P <0.05, **P < 0.01, ***P < 0.001, ****P < .00001. IMC, idomethylcholine.

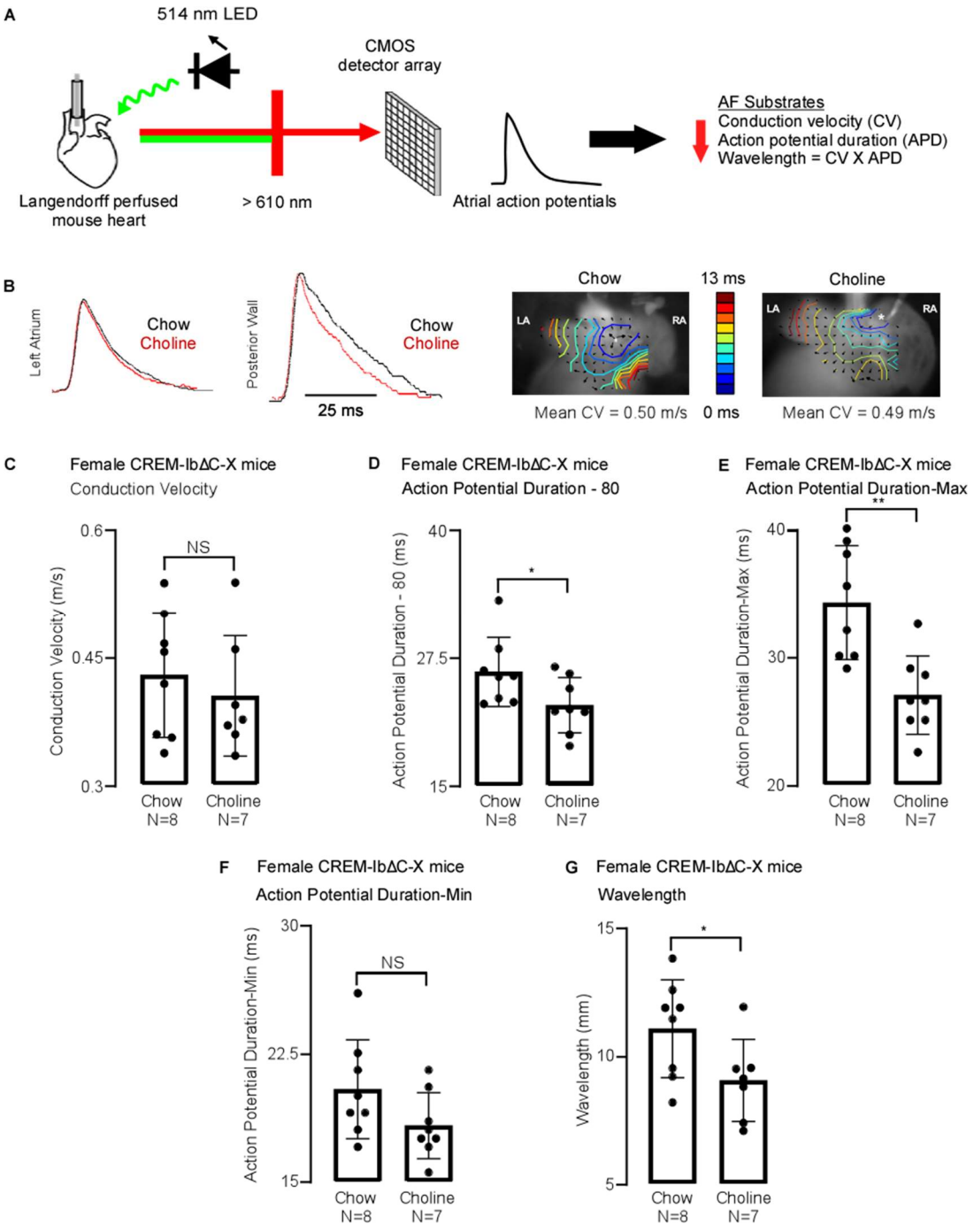

Figure 9: Analysis of conduction velocity (CV) and action potential duration (APD) in CREM-IbΔC-X mouse hearts using *ex vivo* optical mapping on chow vs choline supplemented diets. (**A**) Schematic diagram of the optical mapping experimental setup used for whole heart atrial action potential measurements (see text for details). Representative examples (**B**) of action potentials (left) measured from the LA and posterior wall (PW) for chow (black) and choline (red), and atrial activation maps (posterior view) duing 130 ms pacing (*) with local CV vectors superimposed for chow (middle) and choline (right). Grouped data of (**C**) CV (**D**) Action Potential Duration 80, (**E**) Action Potential Duration 80 maximum, (**F**) Action Potential Duration 80 minimum, and (**G**) wavelength at a pacing cycle length of 130 ms. In C-G, all *P* values shown were determined using the student's 2-tailed t test to compare the two means of two independent groups, after confirmation of normality using Shapiro-Wilks and Kolmogorov-Smirnov tests. NS, Non-Significant, **P* <0.05, ***P* <0 .01.

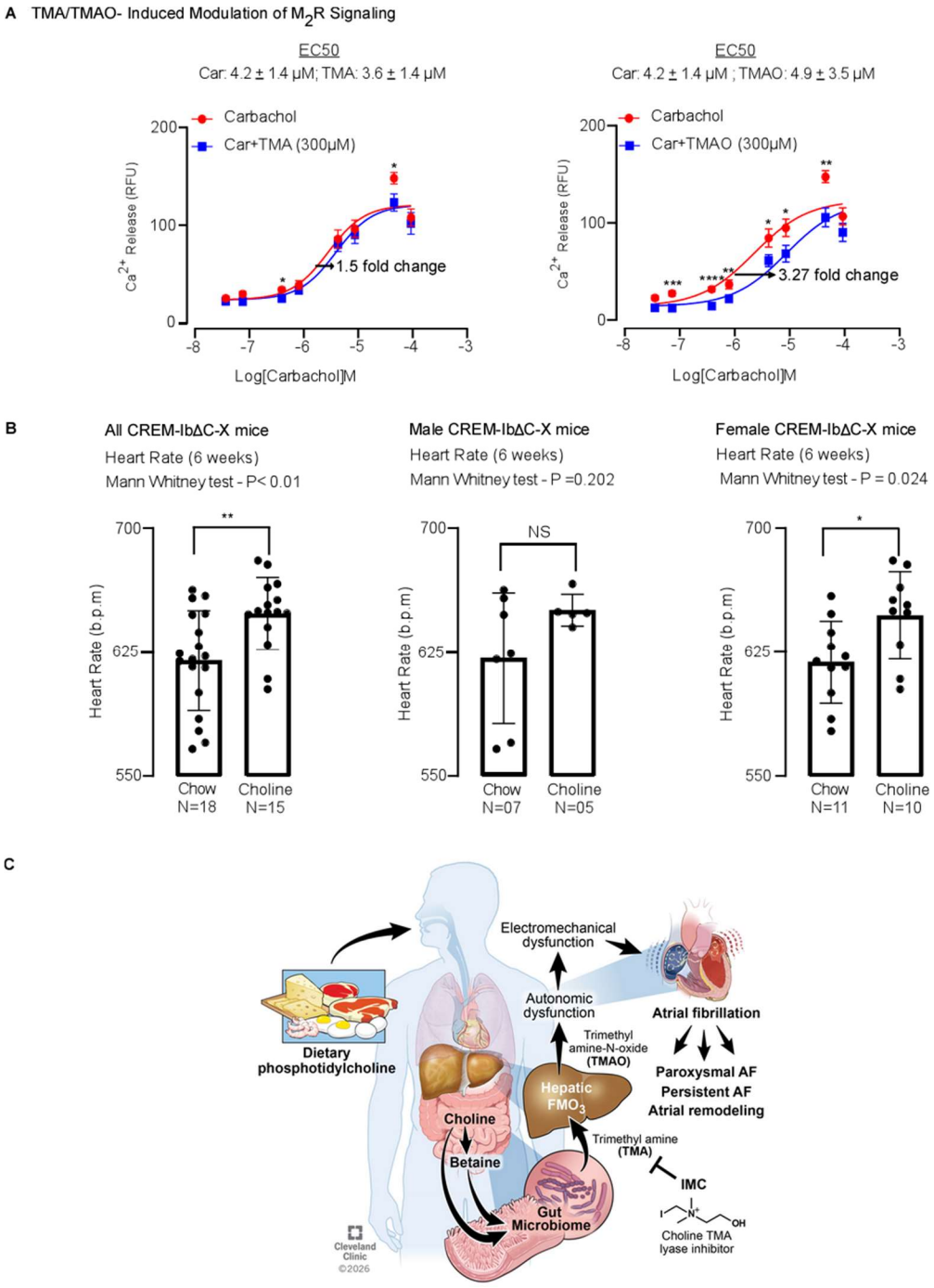

Figure 10: TMAO modulates autonomic signalling. (**A**) TMA/TMAO induced inhibition of M_2R (Clone-1) signalling in HEK293 cell. Two-tailed unpaired Welch's student t-test was used to obtain the fold change P-value by comparing the mean EC50 values of Car and TMA/TMAO (n= 6 independent experiments). (**B**) Heart rate in CREM-IbΔC-X mice treated with choline and chow diet. Each box plot represents mean ±SD. A Mann Whitney test was used to assess significant differences between groups. **P* <0.05, ***P* < 0.01, NS, Non-Significant. (**C**) Scheme demonstrating that IMC inhibits TMA/ TMAO formation, AF induction, and progression. Choline diet alters gut microbial TMA and its metabolite formation. TMAO formation plays a key role in autonomic dysfunction via inhibition of the M_2R and resulting increasing sympathetic tone and susceptibility to AF. IMC that Inhibits formation of TMA/TMAO can reduce AF induction and progression. AF, atrial fibrillation; Car, carbachol; IMC, idomethylcholine; M_2R, muscarinic 2 receptor; HEK293, Human Embryonic Kidney293; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.